



Attorney's Docket No.: 07252-008001

#16128
1/7/03

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Zyskind, J. W., et al.
Serial No. : 08/971,090
Filed : November 14, 1997
Title : METHOD FOR IDENTIFYING MICROBIAL PROLIFERATION GENES

Art Unit : 1655
Examiner : Jeffrey Fredman, Ph.D.

TECH CENTER 1600/2900

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.131

Sir:

1. I, R. Allyn Forsyth, am a co-inventor with Judith W. Zyskind, on the above-identified patent application, which was filed on November 14, 1997.

2. Judith W. Zyskind and I conceived of the claimed invention and reduced it to practice in the United States of America prior to February 14, 1997, which is the filing date of U.S. patent application serial no. (USSN) 08/800,664, which is the priority document for U.S. Patent No. 5,955,275, filed March 4, 1997, and issued on September 21, 1999.

3. As evidence that Judith W. Zyskind and I conceived of the claimed invention and reduced the claimed invention to practice prior to February 14, 1997, I have enclosed as Exhibits A through C, photocopies of laboratory notebook pages, the dates of which have been redacted, but which are prior to February 14, 1997:

(a) Exhibit A contains a copy of one notebook page summarizing the results of experiments practicing a method of the invention that identifies microbial proliferation genes by introducing exogenous nucleic acids into a microorganism, the exogenous nucleic acid having substantial sequence identity to an endogenous microbial gene wherein the exogenous nucleic

CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

Date of Deposit

Signature

Typed or Printed Name of Person Signing Certificate

acid is a random fragment or a random sequence; and identifying the endogenous gene as a microbial proliferation gene by comparing the proliferation or viability of the microorganism when the exogenous nucleic acid is expressed in or introduced into the microorganism with the proliferation or viability of the microorganism when the exogenous nucleic acid is not present or not expressed. Specifically, plasmid constructs containing random fragments of a bacterial genome (an *E. coli* genome) as inserts under the control of an inducible promoter were constructed. The inducible promoter is induced by "IPTG," a chemical that induces expression of the insert under the control of the promoter. The technique wherein random fragments are generated, cloned and transfected is designated as a "shotgun cloning strategy." These plasmids were moved into *E. coli*. The ability of the transfected bacteria to proliferate before and after induction of the inducible promoter was tested. A plasmid was identified that, when expressed in bacteria, inhibited growth of the microorganism after application of IPTG to induce expression of the gene fragment inserts. This plasmid, designated pJB3, was isolated, and the expressed insert was sequenced. This indicated that the insert comprised a fragment of an *E. coli* gene, designated *secA*, and that the insert had been expressed in the sense orientation. The gene *secA* is an essential growth gene in *E. coli*.

(b) Exhibit B contains copies of the notebook pages expressly referred to in the laboratory notebook page contained in Exhibit A: page 64B and pages 71B to 73B.

(c) Exhibit C contains copies of notebook pages numbered 93 to 95, describing experiments similar to those described above in paragraph (a). Page 94 illustrates a summary of this set of experiments. As above, bacterial growth and viability before and after induction of the inducible promoter were tested. Three plasmids were found to inhibit growth and decrease the viability of the bacteria upon induction/ activation of their gene fragment inserts. As above, these plasmids were isolated, and the inserts of these plasmids were sequenced. The plasmid designated pJB3, the same as discussed in paragraph (a), showed significant negative effects on growth and viability. The two inserts from the plasmids designated pJB37 and pJB57, when expressed, also showed significant negative effects on growth and viability. Analysis of the pJB37 insert indicated that it was a gene expressed in a sense orientation and that, specifically, it

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was a 546 base pair *PstI/HindIII* fragment of *lepA* (see declaration by Dr. R. Allyn Forsyth submitted with response dated June 4, 1999, for the instant application). Analysis of the pJB57 insert indicated that it comprised at least two genes: one gene, a 216 base pair *BamHI* fragment of *tsf* and *rspB* expressed in a sense orientation; and, a second gene, a 176 *EcoRI/BamHI* fragment of *ddlB* expressed in an antisense orientation (see declaration by Dr. R. Allyn Forsyth, dated May 3, 1999). These genes (*lepA*, *tsf* and *rspB*, and *ddlB*), identified by the methods of the claimed invention, are essential for bacterial growth and viability.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted

Date: 10/26/2000

R. Allyn Forsyth
R. Allyn Forsyth

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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(a) Exhibit A contains a copy of one notebook page summarizing the results of experiments practicing a method of the invention that identifies microbial proliferation genes by introducing exogenous nucleic acids into a microorganism, the exogenous nucleic acid having substantial sequence identity to an endogenous microbial gene wherein the exogenous nucleic acid is a random fragment or a random sequence; and identifying the endogenous gene as a microbial proliferation gene by comparing the proliferation or viability of the microorganism when the exogenous nucleic acid is expressed in or introduced into the microorganism with the proliferation or viability of the microorganism when the exogenous nucleic acid is not present or not expressed. Specifically, plasmid constructs containing random fragments of a bacterial genome (an *E. coli* genome) as inserts under the control of an inducible promoter were

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Respectfully submitted

Date: _____

August 4, 2000

Judith W. Zyskind

Judith W. Zyskind

**APPENDIX A
TO
DECLARATION OF JUDITH W. ZYSKIND**

10047539.doc

Produced: or [redacted] shotgun cloning strategy

Size: 4.6 Kb [redacted]

Insert: ≈ 850 bp (vec = 4.1~4.2) ~~EcoRI~~ & PstI

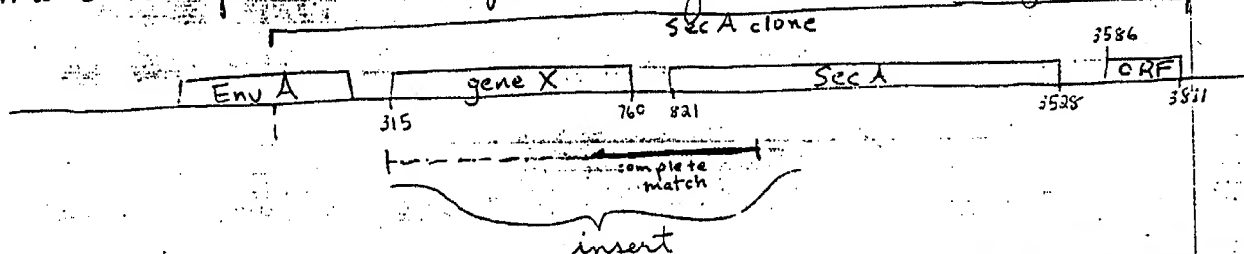
Sensitivity to IPTG:

- on [redacted] IPTG growth experiment conducted (pg 64) B lab notes
- & pJB3 showed sensitivity
- other viable cell count experiments pgs 71-73 shows sensitivity as well (9.8×10^8 viable; control = 9.8×10^8)

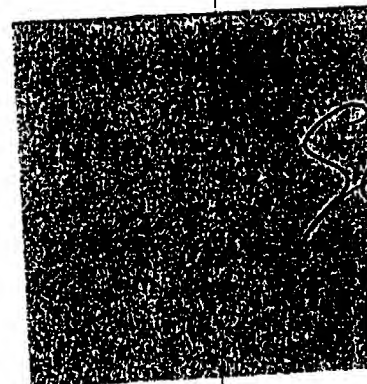
Sensitivity on Replica plates: no growth; revertant colonies day #2

* pJB3 showed exact size of clones 2 & 7; even when cut w/a third Restriction Enzyme Sau3A1.

Insert components: NCBI data bases / genbank
- matched up with a portion of the Sec A gene

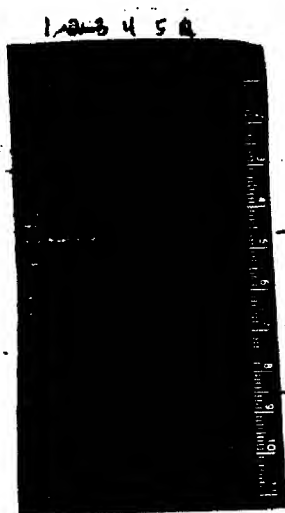


paragraph
3a



Here I 2 4 mutants to 2 nine by RE digest because of their initial size (about 4.8-5.5K

1hr 45min
@ 75 mA¹⁵
5% TEA
agarose
stained
for OR.

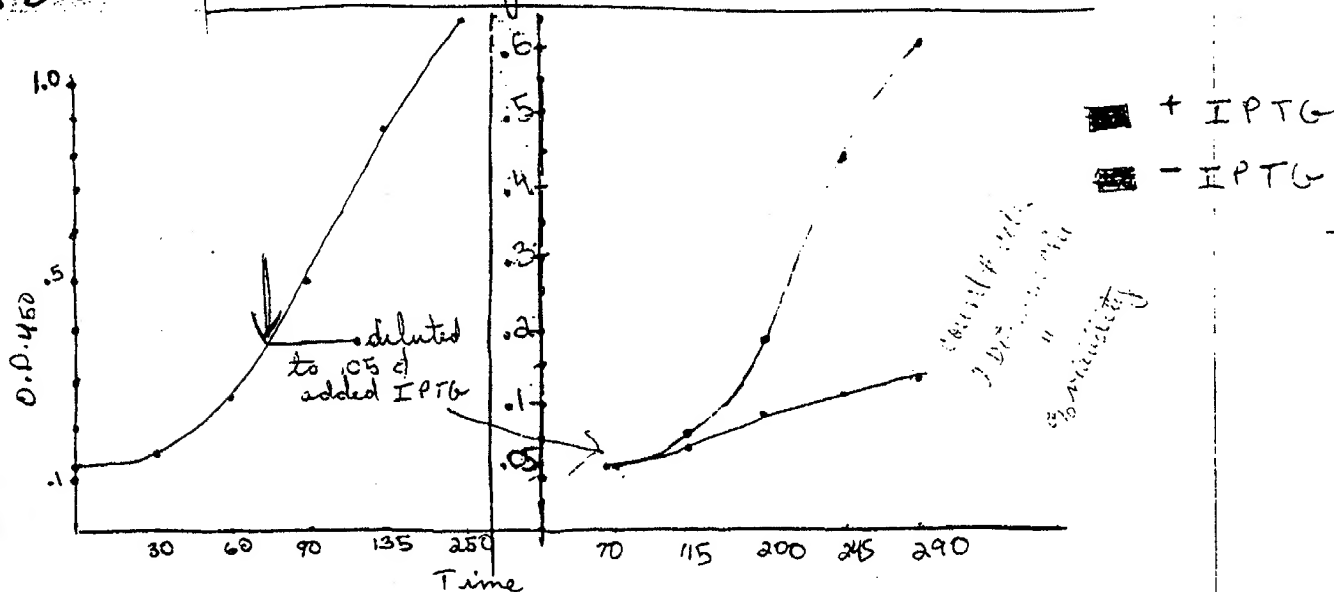


- 1) 1 Kb ladder 21
- 2) p5B19 cut w/ Hind III & Pst I 101
- 3) " 12 " " " " " } 100
- 4) " 13 " " " " " }
- 5) " 16 " " " " " }

Results: All had complete cutting but there doesn't seem to be any inserts released that are 1Kb. The smaller ~~these~~ inserts are lanes 3 (900bp) & 4 (900bp).

Paragraph
3b

Evidence of IPTG sensitive cells



HL5α cells w/ p5B13 were grown in LB at 37°C w/ Ap¹⁰⁰. Here, graphs show growth + & - IPTG. ~~the~~ cells were induced w/ IPTG after 70 min of growth to O.D.₄₅₀ of 0.5, then grown for another 220 min. The slow growth of IPTG+ cells helps our decision to get p5B13 sequence.

quencing clone 3

grew pJBI3 ON in 5 ml LB + $4p^{100}$

- diluted back 1/200 (100mls LB + $4p^{100}$)

- @ $OD_{450} = .05$ - moved 50 mls to sidearm flask w/ IPTG
 & " " " " to sidearm flask w/ 100 μ M IPTG

- every 30' remove 1 ml of each culture; dilute out to 1×10^8
 and plate on Ap^{100} + 100 μ M IPTG & Ap^{100} only

	-IPTG	+IPTG
T_0	.07	.06
T_{30}	.144	.13
T_{60}	.27	.21
T_{90}	.44	.28
T_{120}	.60	.36 *
T_{150}	.85	.44 *
T_{180}	.97	.46
T_{210}	.75(2)	.5
T_{240}	.85(2)	.58
340	0.72(4)	.7
930am	(47) ^{3.76} 8	(43) ^{1.23} 4 *
T_{1360}		

no plating

no plating

Plating:

NI = noninduced
 I = induced w/ 100 μ M IPTG

T_0	NI (10^{-5} , 10^{-6} , 10^{-7}) on \pm IPTG
	I (" , " , ") on \pm IPTG
$T_{30,60}$	NI (10^{-6} , 10^{-7} , 10^{-8}) on - IPTG
	I (" , " , ") on \pm IPTG
T_{90}	NI (" , " , ") " " "
	I (" , " , ") " " "
$T_{120,150,180}$	NI (" , " , ") on - IPTG
	I (" , " , ") on \pm IPTG
T_{210}	NI (" , " , ") " " "
	I (" , " , ") " " "

* 10^{-7} dilution was incorrect 100 μ culture / 990 $\approx 10^{-1}$ final dilutions are 10^{-7} , 10^{-8} , 10^{-9}

* collect the following for Westerns

+IPTG culture

$$(3.84 \text{ mls})(1.72 \text{ OD}) = 6.6 \text{ OD mls}$$

$$\frac{6.6 \text{ OD}}{V_T} = 50 \text{ O.D. } V_T = 132 \text{ ul}$$

66ul Solubilization + 66ul 2x Buffer loading

-IPTG culture

$$(1.92 \text{ mls})(3.76 \text{ OD}) = 7.22 \text{ OD mls}$$

$$\frac{7.22}{V_T} = 50 \text{ O.D. } V_T = 144 \text{ ul}$$

72ul Sol. buffer + 72ul 2x Buffer loading

Materials:

Viable cell count

10
B

- 10 sidearm flasks
w/ 100 mL L.B.
+ Ap^{100}

- 70 Ap^{100} plates
 $T_0 = 40$ plates
 $T_{240} = 30$ plates

- OD₄₅₀ spec.

- IPTG @ 100 μ M
(add 100 μ L / 100 mL LB for
conc. of 100 μ M)

- eppies with serial dil.
setup: $T_0 \rightarrow 10^{-4}, 10^{-5}$

$T_{240} \rightarrow 10^{-2}, 10^{-4}, 10^{-5}$

- turn table

Procedure:

1) dilute all sidearm flasks
1:500 with D.W. 1-10
(200 μ L ON \rightarrow 100 mL L.B. amp)

2) agitate all cultures @ 37°C
until OD₄₅₀ has reached
0.1 - 0.2 on spec.

3) aliquot desired volumes from
all cultures for serial dil.
& plating at T_0 .

ex) 10 μ L culture \rightarrow 990 H₂O = 10^{-2}

$\frac{10^{-2}}{10^2} \rightarrow \frac{10^{-4}}{\text{plate LB amp}} = 10^{-6}$

1. 2 sets
2. independent
3. dilution + plating
4. for culture

5) after 4 hr of 37°C shaking
at T_{240} , aliquot desired
volume of cultures and
do as in step #3

6) put plates in incubator
at 37°C

* in planning to
grow for 15 hr
Too long / short

11) after aliquoting, immed. add IPTG
to fin. conc. of 100 μ M in all
cultures, return to 37°C shaking,
and resume with ser. dil. &
plating at T_0

Cloning counts

*Wanted to check sensitivity of all clones created up to this point in order to see which were worth sequencing

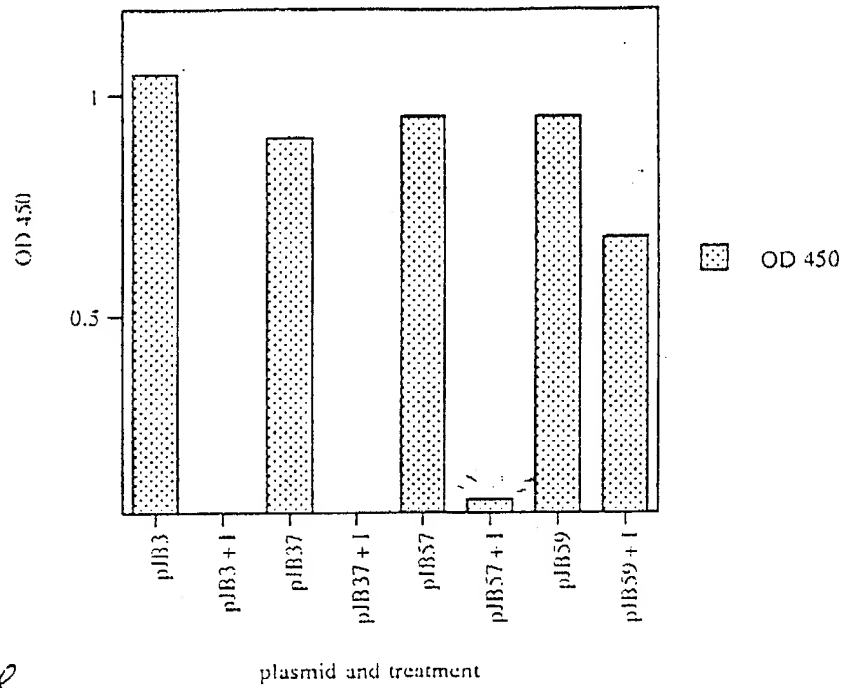
- grew 4 ml O.N.'s of all clones: (in LB+Ap¹⁰⁰ @ 3:
1-28 clones
29-48 subclones > 42 in all

- next day added 100 μ M IPTG & grew for 30 hrs

-
data
→
over

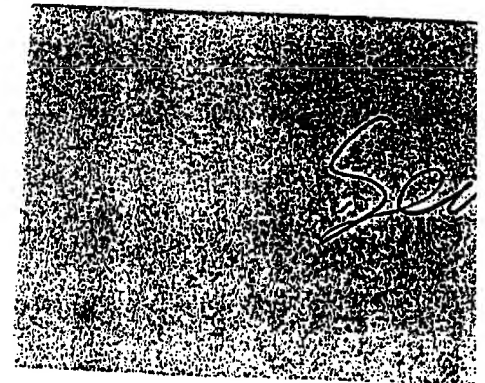
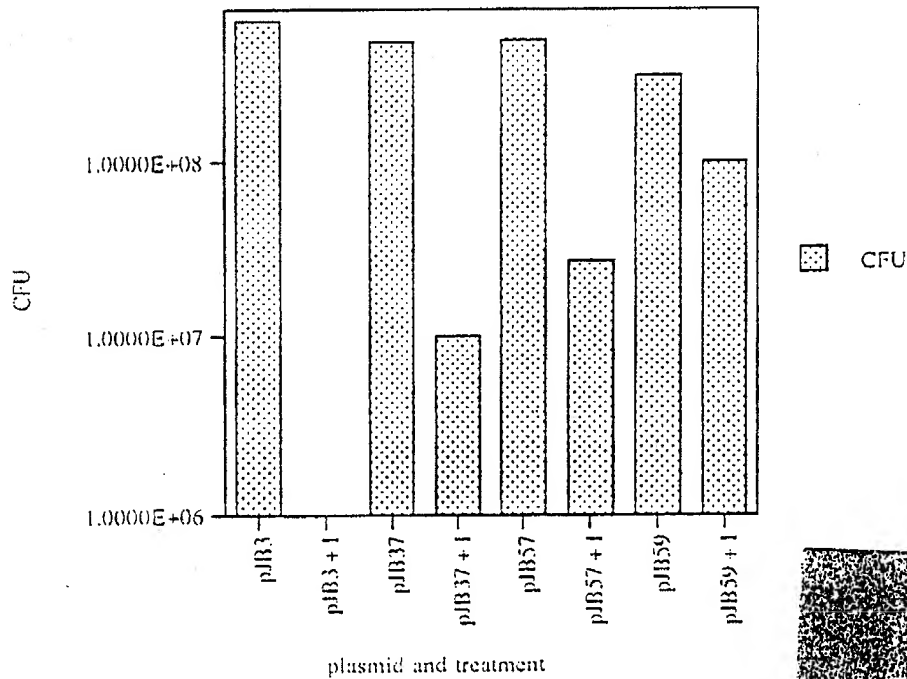
Used Ultra Clean
Kit to purify
vector fragments
of pLEX1-3RS to
push away from Origin NotI fragments.
Resuspended silica pellet in 10 μ l of H_2O used
to resuspend pellets shown in 10/3/96 gel.

Exhibit
Growth of cells with Induction of Antisense RNA



2133 secA sense
37 ~~secA~~ antisense
57 ~~secA~~
59 ampG

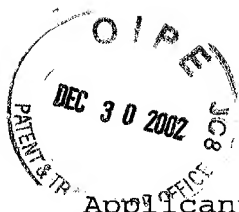
Viability of cells carrying antisense plasmids *with Induction*



CONFIDENTIAL

	1	2	3
	plasmid	OD 450	CFU
1	pJB3	1.05	6.0000E+08
2	pJB3 + I	0	1.0000E+06
3	pJB37	0.9	4.6000E+08
4	pJB37 + I	0.01	1.0000E+07
5	pJB57	0.95	4.7000E+08
6	pJB57 + I	0.08	2.7000E+07
7	pJB59	0.95	3.0000E+08
8	pJB59 + I	0.68	1.0000E+08
9			
10			

Cultures were inoculated Sunday-night (9³⁰pm) $\times 10^8$
 into LBap¹⁰⁰ #100um IPTG. 18 hrs later at 3³⁰pm. OD₄₅₀
 was read and ~~cells~~ cultures were serially diluted and plated
 LBap¹⁰⁰.



PATENT
ATTORNEY DOCKET NO. 07252/008001

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Box AF
Assistant Commissioner for Patents
Washington, DC 20231

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DECLARATION UNDER 37 C.F.R. §1.132 OF R. ALLYN FORSYTH

I, R. Allyn Forsyth, hereby declare as follows:

1. That I am a named inventor on U.S. patent application Serial No.: 08/971,090;

2. That I am presently employed by Elitra Pharmaceuticals, Inc., San Diego, California, as a Research Scientist and have been so employed since 1997;

3. That Elitra Pharmaceuticals is a licensee of the invention claimed in U.S. patent application Serial No.: 08/971,090;

4. That, subsequent to the filing date of U.S. patent application Serial No. 08/971,090, I or individuals under my supervision undertook a recharacterization of the plasmids listed in Table 1 of the application. The investigation revealed that the nature and orientation of the inserted sequences in certain plasmids is as indicated in the Table below.

Date of Deposit June 4, 1999
I hereby certify under 37 CFR 1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Ann Pommier
(Ann Pommier)



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Declaration Under 37 CFR §1.132 of R. Allyn Forsyth
Serial No.: 08/971,090

TECH CENTER 1600/2900

Characteristics of Plasmids
Listed in Table 1 of U.S. 08/971,090

Plasmid ¹	Insert	Orientation
pJB12	172 bp <i>E. coli</i> fragment, about 520 bp <i>E. coli</i> fragment plus about 700 bp fragment of <i>lepB</i>	172 bp: Unknown 520 bp: Unknown <i>lepB</i> : Antisense
pJB37	546 bp <i>PstI/HindIII</i> fragment of <i>lepA</i>	Sense
pJB40	546 bp <i>Pst/Hind III</i> fragment of <i>lepA</i>	Sense
pJB53	714 bp <i>PstI/HindIII</i> fragment of <i>viaA</i>	Sense
pJB57	216 bp <i>BamHI</i> fragment of <i>tsf</i> and <i>rpsB</i> plus 176 bp <i>EcoRI/BamHI</i> fragment of <i>ddlB</i>	<i>tsf/rpsB</i> : Sense <i>ddlB</i> : Antisense
pJB59	144 bp <i>BamHI</i> fragment of <i>ilvC</i> and 451 bp <i>EcoRI/BamHI</i> fragment of <i>orf1/ampG</i>	<i>ilvC</i> : Sense <i>orf1/ampG</i> : Antisense
pcW37	pJB37 insert in reverse orientation	Antisense
pcW53	pJB53 insert in reverse orientation	Antisense
pcW57	pJB57 inserts in reverse orientation	<i>tsf/rpsB</i> : Antisense <i>ddlB</i> : Sense
pcW59	pJB59 inserts in reverse orientation	<i>ilvC</i> : Antisense <i>orf1/ampG</i> : Sense
pAF37	Same insert as pJB37	Sense
pAF53	Same insert as pJB53	Sense
pAF57	Same insert as pJB57	<i>tsf/rpsB</i> : Sense <i>ddlB</i> : Antisense
pAF59	Same insert as pJB59	<i>ilvC</i> : Sense <i>orf1/ampG</i> : Antisense

¹ Inserts in plasmids designated pJB or pcW were cloned in the pLEX5BA vector; inserts in plasmids designated pAF were cloned in the pAF Ω vector.

5. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: May 3, 1999

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